

# Construction of a Mutant pBR322 Using Site-Directed Mutagenesis to Investigate the Exclusion Effects of pBR322 During Co-transformation with pUC19

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It has been observed that when pBR322 and pUC19 plasmids (derivatives of pColE1) are co-transformed the pBR322 plasmid is selectively excluded from the cell. There are several potential factors that may explain this observation. One particular factor may be that pBR322 encodes the gene for the Rop protein, not seen in pUC19. This protein is involved in stabilizing the interaction between RNA I and RNA II, which in turn prevents the replication of pBR322. The focus of this study was to create an altered pBR322 plasmid such that it contains a mutation in the ribosome binding site. In theory, a mutation in the ribosome binding site would lead to a decreased production of the Rop protein, which may result in a higher copy number pBR322 and a reduction in exclusion effect during co-transformation with pUC19. Site-directed mutagenesis was used to introduce a mutation in the ribosome binding site along with a new *Alu I* restriction site not present in wild-type pBR322. Following mutagenic PCR, the mixture was transformed into *Escherichia coli* DH5 $\alpha$  cells and five colonies were chosen for restriction digests. All five colonies showed a consistent restriction pattern, which was different from the wild-type pBR322 plasmid. Future work will confirm the identities of these colonies, and co-transformation tests will assess the role of Rop.

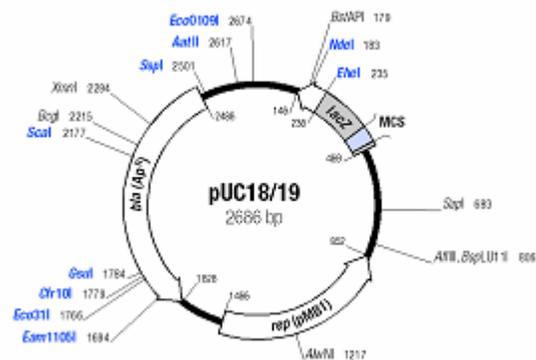
Plasmids pBR322 and pUC19 (figure 1) are two very common vectors that are widely used for the cloning of recombinant DNA in bacteria. Both of these plasmids are derivatives of another plasmid known as pColE1 (2).

When the plasmids, pBR322 and pUC19, are co-transformed into the same cell an exclusion effect is observed such that pBR322 is selectively eliminated from the cell (5). Several explanations have been offered for this observation. One factor that may play a role in this exclusion effect is the size difference in these two plasmids. pBR322 (4361 bp) is significantly larger than pUC19 (2686 bp), and as a result, its replication rate is lower than that of pUC19. As the cells are replicating, the replication may not be able to keep up with the rate of division, and the plasmid would eventually be eliminated (5).

Another factor that may explain the exclusion of pBR322 in this co-transformation experiment is the presence of the *rop* gene on pBR322, which is not seen in pUC19. In pColE1-type plasmids, the replication is controlled via two RNA molecules, RNA I and RNA II (2). Plasmid replication is initiated by RNA II, which undergoes RNase H cleavage and provides a 3'OH end that is able to be used by the DNA polymerase to start replicating. RNA I is complementary to RNA II and, therefore, is able to bind to RNA II, inhibiting initiation of replication. This interaction between RNA I and RNA II is stabilized by the Rop protein, which is

encoded on the pBR322 plasmid (2). The *rop* gene encodes the Rop protein, which, as mentioned earlier, stabilizes the interaction between RNA I and RNA II and inhibiting plasmid replication. pUC19 does not express the Rop protein, allowing RNA I and RNA II to dissociate more frequently (3, 5).

Finally, a single point mutation is observed in the sequence for the RNA II transcript. This mutation inhibits the interaction between the two RNA molecules, allowing RNA II to initiate plasmid replication of the pUC19 plasmid. This mutation is not seen in the pBR322 plasmid. As a result, the replication of pBR322 is relatively reduced due to the interaction of the two RNA molecules (2, 5).





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      A G T
AAATCCCCCTTACACGGAGGCATCAGTGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATC
AGAA GCCAGACATTAACGCTTCTGGAGAAACTCAACGAGCTGGACGCGGATGAACAGGCAGACATCTGTG
AATCGCTTACGACCACGCTGATGAGCTTTACCGCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGA AAA
CCTCTGA

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**FIG. 2** Sequence from GenBank (J01749) showing the *rop* gene (underlined, 1915-2106bp), and the ribosome binding site (dark shading, 1905-1909 bp). The 1890-2106 bp sequence is shown in the 5' to 3' direction. The light shading corresponds to the region of the pBR322 plasmid for which the mutagenic PCR primers were designed. The mutations to be incorporated into the sequence using site-directed mutagenesis are shown above the sequence. The three base substitutions are intended for the mutagenesis of the ribosome binding site as well as for the creation of an *Alu* I restriction enzyme site, which is used to determine if the mutagenesis was successful.

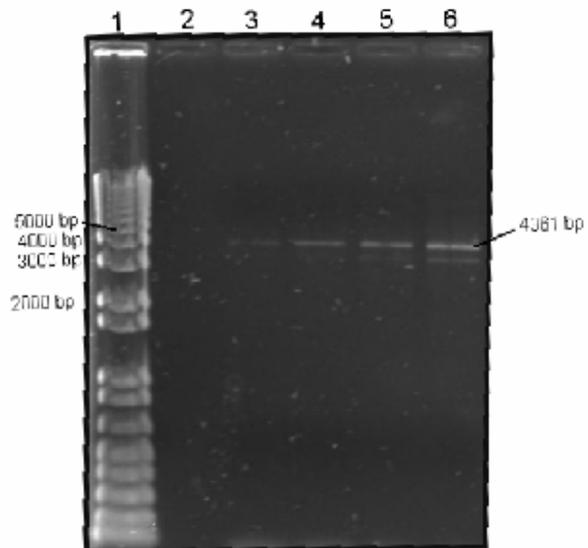
**Transformation.** Following the overnight digestion with *Dpn* I, the PCR mixture was used to transform competent *E. coli* DH5 $\alpha$  cells, and the sample was plated onto LB + ampicillin (100  $\mu$ g/mL) plates. The competent cells were prepared by another student in the previous year and stored at -80°C. Two microliters of the PCR mixture containing 50 ng of the original template pBR322 was used in the transformation. A positive control was carried out using 240 ng of wild-type pBR322 plasmid. The competent DH5 $\alpha$  cells were thawed on ice, and 50  $\mu$ L of the DH5 $\alpha$  cells were added to the PCR mixture and to the wild-type plasmid. This was incubated on ice for 30 minutes. The cells were heat shocked at 41°C for 45 seconds, and placed on ice for 2 minutes. Eight hundred microliters of sterile LB broth was added to each mixture and the samples were transferred into sterile glass tubes and incubated in a shaker at 37°C for 1 hr. Following the incubation, samples were transferred to Eppendorf tubes and centrifuged at 3000 rpm for 3 minutes. Seven hundred and fifty microliters of the broth was discarded following centrifugation, and the cells were re-suspended in the remaining 100  $\mu$ L LB broth. Fifty microliters were spread plated on LB plates containing 100  $\mu$ g/mL of ampicillin. The plates were then incubated at 37°C overnight. The following day, nine colonies were observed, and five colonies were re-streaked on fresh LB + ampicillin plates. They were then grown in 5 mL LB broth containing 100  $\mu$ g/mL ampicillin. The plasmids were then isolated from each of the five grown cultures using the Qiagen QIAprep Spin Miniprep Kit (cat# 27104) according to the instruction manual.

**Restriction digests.** The restriction digests were carried out on the PCR products as well as a wild-type pBR322 in order to determine whether there are differences in the digestion patterns for the two plasmids. Plasmids isolated from five different strains (IK051, IK052, IK053, IK054 and IK055), along with the wild-type pBR322, were digested with *Alu* I (Fermentas, cat# ER0011, lot# 4311) at 37°C overnight. The digests were carried out in a final volume of 25  $\mu$ L with the following components: 1X Buffer Tango (supplied with *Alu* I), 2 U of *Alu* I and 1

$\mu$ g wild-type or mutated pBR322 plasmid DNA. The digest mixtures were then separated on a 2% agarose gel to determine whether the site-directed mutagenesis was successful. Twenty microliters of 100 bp standard (Invitrogen, cat# 10380-012, lot# 1030296) and 10  $\mu$ L of 100 bp standard (Fermentas, cat# SM0248, lot# 1401) were also run on the 2% agarose gel.

## RESULTS

**Site-directed mutagenesis PCR.** The pBR322 plasmid isolated from *E. coli* DH5 $\alpha$  cells was used as the original template for the site-directed mutagenesis PCR. Following the site-directed mutagenic PCR, a PCR product was observed on a 1% agarose gel in four samples which contained different starting amounts of template DNA (figure 3).



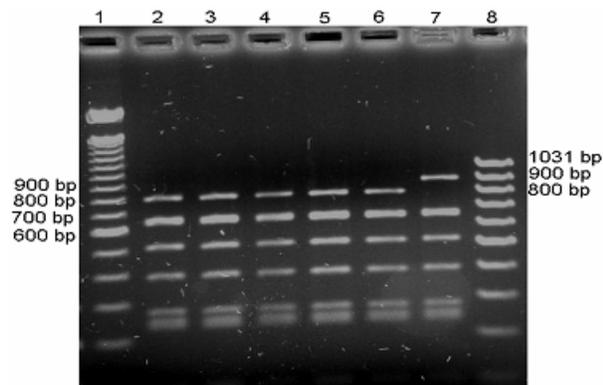
**FIG. 3** Results from the site-directed mutagenesis PCR. Lane 1 contains the 1 Kb Plus DNA standard. Lanes 3-6 contain the four PCR products. The amount of DNA used for PCR in lanes 3, 4, 5 and 6 was 5 ng, 10 ng, 20 ng and 50 ng respectively.

The sample containing 50 ng of template pBR322 showed the most intense band (figure 3). The molecular weight of the band observed is also the expected size of the pBR322 plasmid, which is 4361 bp.

### Transformation and plasmid isolation.

Following transformation nine colonies were observed, and five colonies were randomly chosen for restriction digests with *Alu* I. These five colonies were named IK051, IK052, IK053, IK054 and IK055.

**Restriction digests.** The five randomly chosen colonies were digested with *Alu* I, and the samples were run on a 2% gel to ensure full separation of all bands. There appears to be some differences in the digestion patterns of the wild-type pBR322 and the mutated PCR pBR322 product from all five colonies that were tested (figure 4).



**FIG. 4** Photograph of a 2% agarose gel loaded with the restriction digest mixtures. Lane 1 contains the Gibco 100 bp DNA standard. Fermentas' 100 bp DNA ladder is shown in lane 8. Lanes 2 through 6 contain the digested mutant pBR322 plasmid, and the digest of the wild-type pBR322 is shown in lane 7.

Lane 7, which contains the wild-type pBR322, has a band with an approximate molecular weight of 900 bp that is not observed in any of the five mutated plasmids in lanes 2-6. In lanes 2 through 6, a band with an approximate molecular weight of 800 bp is observed. The remaining bands appear to be identical in both mutated and wild-type pBR322 plasmids.

### DISCUSSION

Using the site-directed mutagenesis method, a pBR322 with a mutation in the ribosome binding site was created. Mutagenic primers were designed so that both the reverse and forward primers anneal to opposite strands on the same region of the plasmid. Both primers contained three base substitutions, which when incorporated into the newly synthesized pBR322 would lead to the desired mutation in the ribosome binding site as well as a new *Alu* I restriction site. At position 1905, the substituted base is required for mutating the ribosome binding site for the *rop* gene in pBR322 as well as to introduce a restriction site for the endonuclease *Alu* I, which has the sequence AGCT. The mutations at positions 1902 and 1903 are also

required to complete this restriction site. The primers used for this PCR reaction are shown in figure 2, and the intended base substitutions are also shown above the sequence of the *rop* gene. The PCR was carried out with a low number of cycles to prevent the formation of a large catenated product, as there is no enzyme present to resolve these types of structures (3). The low number of cycles was suggested by the Stratagene QuikChange® Site-Directed Mutagenesis Kit (4). Perhaps the most important factor that contributed to the success of this PCR is the *Pfu* polymerase enzyme. This particular polymerase has six fold higher fidelity in DNA synthesis than does the *Taq* DNA polymerase (4). In addition, the resulting PCR product contains nicked circular strands, which could be another factor that contributed to the success of the PCR (4). The nicks in the circular DNA may be helpful in preventing the entangling of the resulting product. In doing so, it would allow the circular plasmids to dissociate from the template DNA so that replication can continue to occur. Previous studies have shown that replication of an entire plasmid using *Taq* DNA polymerase does not result in a visible PCR product (3). The author's explanation for this result is that the polymerase does not have a mechanism of dissociating the replicated DNA strands, which causes the DNA to become tangled (3). This problem was not encountered with the *Pfu* DNA polymerase, and a PCR product with the correct molecular weight was observed on an agarose gel.

The resulting PCR product was then treated with *Dpn* I endonuclease. The purpose of this treatment is to remove any parental DNA template from the PCR mixture. *Dpn* I is specific for methylated and hemimethylated DNA and will selectively digest any parental DNA (4), which in this case is the wild-type pBR322. The strain of *E. coli* from which the pBR322 plasmid is isolated from is *dam*<sup>+</sup> and results in the production of methylated pBR322. The PCR product synthesized by *Pfu* is not methylated, and as a result is not susceptible to *Dpn* I digestion. This selective digestion is very useful because it removes any non-mutated plasmids and allows transformation into bacteria with only the mutated pBR322. The PCR product is shown in figure 3. Various amounts (5ng, 10ng, 20ng and 50ng) of parental DNA were used in order to determine the optimum amount of starting template. The results suggest that 50 ng provides an adequate amount of starting material for the PCR. The obtained PCR product had the same approximate size as the wild-type pBR322 plasmid, which is 4361 bp (figure 3).

The *Dpn* I digested PCR product was then transformed into competent *E. coli* DH5a cells in order to generate a larger quantity of the plasmid as well as to repair the nicks in the mutated pBR322. During PCR,

the *Pfu* polymerase extends and incorporates the mutagenic primers giving rise to nicked circular strands (4). Following transformation the cells were plated on LB + ampicillin plates, and nine colonies were observed the following day. Five of these transformed colonies were randomly chosen for restriction digests with *Alu* I.

The *Alu* I digest was intended to determine whether the mutagenesis was successful. The wild-type pBR322 plasmid has 18 *Alu* I restriction sites, and the largest fragment created by digesting the wild-type pBR322 using *Alu* I is 908 bp long. This information was determined by entering the entire pBR322 sequence into the New England BioLabs Cutter (NEB Cutter, www.neb.com), which determines how many *Alu* I restriction sites are present based on the AGCT sequences present. The mutated pBR322, created by site-directed mutagenesis, should contain an extra *Alu* I restriction site, which can be used to determine the success of the mutagenesis. The NEB Cutter was also used to predict the restriction digest pattern for the mutant pBR322 plasmid. It was determined that the largest fragment produced by digesting the mutant pBR322 with *Alu* I would be 813 bp in size. This is different from the largest fragment produced by the wild-type pBR322 (908 bp), which makes it possible to differentiate between wild-type and mutant pBR322.

The results from the *Alu* I digests are shown in figure 4. Lane 7 contains the *Alu* I digest that was carried out on the wild-type pBR322 plasmid. The largest band observed appears to be approximately 900 bp in size, which is the expected size predicted by the NEB Cutter program. The digest pattern observed in the mutant pBR322 plasmid (lanes 2-6) differs from the wild-type pBR322 in that it contains a band of approximately 800 bp in size. This 800 bp band, which is observed among all five samples tested would result from the incorporation of an additional *Alu* I restriction site during site-directed mutagenesis. The presence of an additional *Alu* I restriction site in the mutated pBR322 indicate that the site-directed mutagenesis was successful.

The presence of an additional *Alu* I restriction site indicates that the desired mutation in the ribosome binding site was also obtained. The primers were designed so that the mutation in the first base of the ribosome binding site (GGAGG) is also a mutation that is required for the creation of the *Alu* I restriction site. This means that a pBR322 plasmid with a mutation in the first position of the ribosome binding site was successfully created.

The original experiment included a co-transformation experiment in order to observe the effects the mutation has on the exclusion and copy number of pBR322. The wild-type pBR322, which has a normal expression of the Rop protein, is selectively

excluded from bacterial cells following co-transformation with pUC19 (5). So in theory, the mutant pBR322, which should have a decreased expression of the Rop protein, should result in higher copy number and a reduction in exclusion of pBR322 during co-transformation with pUC19. Unfortunately, these experiments could not be carried out due to a lack of time.

## FUTURE EXPERIMENTS

There are several experiments that could be carried out using the mutant pBR322 plasmid that was created in this study. One experiment that may help to determine the role of the Rop protein is the co-transformation experiment using pUC19 and the mutant pBR322 created in this study. The results can then be used to compare to results produced in a co-transformation experiment using pUC19 and wild-type pBR322. The theory is that if the exclusion effect is due to the presence of an expressed *rop* gene on the pBR322 plasmid, then by decreasing its expression we should observe a higher pBR322 copy number and reduced exclusion during co-transformation.

This experiment could be done by co-transforming *E. coli* DH5 cells with various amounts of pUC19 and pBR322 and plating them on different media for differential selection of plasmids. The transformation can be repeated using the wild-type pBR322 and keeping the amounts of plasmids equal so that direct comparisons can be made with the mutant pBR322. These results can then be used to determine if there is a difference in exclusion and copy number between the wild-type and mutant pBR322.

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